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*Published in:*  
Applied and environmental microbiology

*DOI:*  
[10.1128/AEM.00052-18](https://doi.org/10.1128/AEM.00052-18)

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*Document Version*  
Publisher's PDF, also known as Version of record

*Publication date:*  
2018

[Link to publication in University of Groningen/UMCG research database](#)

### *Citation for published version (APA):*

Li, Q., Montalban-Lopez, M., & Kuipers, O. P. (2018). Increasing Antimicrobial Activity of Nisin-based Lantibiotics Against Gram-negative Pathogens. *Applied and environmental microbiology*, 84(12), [UNSP e00052-18]. <https://doi.org/10.1128/AEM.00052-18>

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# Increasing the Antimicrobial Activity of Nisin-Based Lantibiotics against Gram-Negative Pathogens

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**ABSTRACT** Lantibiotics are ribosomally synthesized and posttranslationally modified antimicrobial compounds containing lanthionine and methyl-lanthionine residues. Nisin, one of the most extensively studied and used lantibiotics, has been shown to display very potent activity against Gram-positive bacteria, and stable resistance is rarely observed. By binding to lipid II and forming pores in the membrane, nisin can cause the efflux of cellular constituents and inhibit cell wall biosynthesis. However, the activity of nisin against Gram-negative bacteria is much lower than that against Gram-positive bacteria, mainly because lipid II is located at the inner membrane, and the rather impermeable outer membrane in Gram-negative bacteria prevents nisin from reaching lipid II. Thus, if the outer membrane-traversing efficiency of nisin could be increased, the activity against Gram-negative bacteria could, in principle, be enhanced. In this work, several relatively short peptides with activity against Gram-negative bacteria were selected from literature data to be fused as tails to the C terminus of either full or truncated nisin species. Among these, we found that one of three tails (tail 2 [T2; DKYLPRPRPV], T6 [NGVQPKY], and T8 [KIAKVALKAL]) attached to a part of nisin displayed improved activity against Gram-negative microorganisms. Next, we rationally designed and reengineered the most promising fusion peptides. Several mutants whose activity significantly outperformed that of nisin against Gram-negative pathogens were obtained. The activity of the tail 16 mutant 2 (T16m2) construct against several important Gram-negative pathogens (i.e., *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*) was increased 4- to 12-fold compared to that of nisin. This study indicates that the rational design of nisin can selectively and significantly improve its outer membrane-permeating capacity as well as its activity against Gram-negative pathogens.

**IMPORTANCE** Lantibiotics are antimicrobial peptides that are highly active against Gram-positive bacteria but that have relatively poor activity against most Gram-negative bacteria. Here, we modified the model lantibiotic nisin by fusing parts of it to antimicrobial peptides with known activity against Gram-negative bacteria. The appropriate selection of peptidic moieties that could be attached to (parts of) nisin could lead to a significant increase in its inhibitory activity against Gram-negative bacteria. Using this strategy, hybrids that outperformed nisin by displaying 4- to 12-fold higher levels of activity against relevant Gram-negative bacterial species were produced. This study shows the power of modified peptide engineering to alter target specificity in a desired direction.

**KEYWORDS** nisin, outer membrane, Gram-negative pathogens, antimicrobial peptide, antimicrobial activity, lantibiotic

**Received** 10 January 2018 **Accepted** 29 March 2018

**Accepted manuscript posted online** 6 April 2018

**Citation** Li Q, Montalban-Lopez M, Kuipers OP. 2018. Increasing the antimicrobial activity of nisin-based lantibiotics against Gram-negative pathogens. *Appl Environ Microbiol* 84:e00052-18. <https://doi.org/10.1128/AEM.00052-18>.

**Editor** Marie A. Elliot, McMaster University

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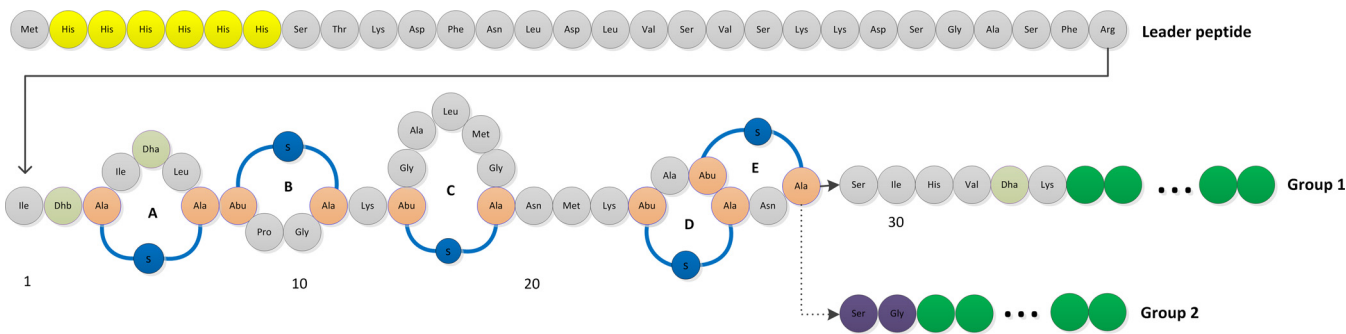
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Nisin (Fig. 1), produced by *Lactococcus lactis*, is the oldest known (since 1928) and most extensively studied lantibiotic (1, 2). It is a potent lanthionine-containing antimicrobial peptide that is ribosomally synthesized and posttranslationally modified. Unmodified prenisin contains 57 amino acids, of which the first 23 amino acids correspond to the leader peptide and the last 34 residues compose the core peptide (2). The leader peptide is crucial for unmodified prenisin to be recognized by the modification and transport proteins (2–4). The precursor is processed by the modification machinery. First, the serine and threonine residues are dehydrated by the NisB dehydratase to become dehydroalanines (Dha) or dehydrobutyrines (Dhb), respectively (2, 3). Dehydrated residues can then be coupled to a cysteine by the cyclization enzyme NisC (5). Subsequently, the modified peptide is transported out of the cell by the transporter NisT (2, 4). At this time, the fully modified nisin prepeptide is still inactive because of the presence of the leader peptide. Only after the leader sequence is cleaved off by the protease NisP does nisin become active and induce the two-component system NisRK (6, 7). It has been clearly demonstrated that NisB, NisC, and NisT have a relaxed substrate specificity, and highly diverse peptides fused to the nisin leader can be efficiently modified (2, 3, 5, 8, 9).

Nisin has been used in the food industry as a natural preservative for decades, thanks to its high level of activity against bacteria and low level of toxicity for humans (2, 10). It is highly effective against Gram-positive bacteria, with its MICs being at nanomolar concentrations (1, 2, 5). There are two different mechanisms by which nisin kills bacteria: pore formation in the membrane and inhibition of cell wall biosynthesis by binding to lipid II (1, 11, 12). After nisin reaches the bacterial plasma membrane, a pyrophosphate cage which involves the first two rings of nisin and the pyrophosphate moiety of lipid II is formed via hydrogen bonds. The pyrophosphate is responsible for the low levels of resistance of bacteria to nisin, since the pyrophosphate is essential and not prone to mutation and also facilitates the transmembrane orientation of nisin (1, 12).

Nevertheless, it is difficult for nisin to penetrate the outer membrane barrier of Gram-negative bacteria, and thus, it cannot reach its target, lipid II, in the inner membrane. This leads to the relatively low level of activity of nisin against Gram-negative bacteria. Conversely, nisin actually tends to bind to the usually anionic surface of the outer membrane and stabilizes it via electrostatic interactions (13). Notably, nisin can inhibit the growth of Gram-negative bacteria more efficiently, when chelating agents (EDTA, citrate monohydrate, or trisodium orthophosphate) are used to destabilize the outer membrane (14, 15). Thus, the main bottleneck for nisin to be active against Gram-negative bacteria appears to be its ability to pass the outer membrane.

The outer membrane of Gram-negative bacteria constitutes an efficient protective barrier that prevents various antimicrobials from reaching the cellular membrane and exerting their function. To address this issue, we have designed hybrid compounds based on the antimicrobial nisin and other antimicrobial peptides that combine different functionalities. Thus, we have developed ways to enable antimicrobials to pass the outer membrane of Gram-negative organisms while retaining as much as possible their antimicrobial function at the cytoplasmic membrane. Some mutants have been made previously (16). In those mutants, full nisin or parts of nisin were fused to different tails with reported activity against Gram-negative bacteria to create antimicrobial peptides targeted against Gram-negative pathogens (16). These tails are mainly cationic peptides secreted by amphibians, insects, or immune cells, including proline-rich antimicrobial peptides (Table 1). Their mechanisms of action are unclear in many cases, but to reach their targets, it is clear that they must interact with and cross the outer membrane. Because peptide GNT16 was reported by our group to have 2-fold improved activity against *Escherichia coli* compared with that of nisin alone (16), in this work the tail 16 (T16) sequence PRPPHPRL was also used for further engineering. In addition, we chose several Gram-negative pathogens from the *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species (ESKAPE) group of pathogens as well as *Escherichia*



**FIG 1** Schematic structure of prenisin with a His tag and fusions. Dha, dehydroalanine; Dhb, dehydrobutyrine; Ala-S-Ala, lanthionine; Abu-S-Ala,  $\beta$ -methylanthionine. Prenisin (gray) contains a leader peptide and a core peptide (1 to 34 amino acids). The 6 histidine residues are located at the N terminus and labeled in yellow. The ABCDE rings are marked. The structures of fusion peptides are indicated, with the linker being labeled in purple (serine and glycine), while tails with activity against Gram-negative bacteria are labeled with green. Group 1 contains full nisin and tails with activity against Gram-negative bacteria. Group 2 contains the ABCDE rings of nisin, the hinge region (serine and glycine), and tails with activity against Gram-negative bacteria.

*coli* as indicator strains for MIC tests. In the course of the experiments, we did a further rational design of the peptides on both the tail and the nisin parts. We found that several hybrid peptides had considerably higher activity than nisin against different multidrug-resistant (MDR) Gram-negative pathogens. The tail 16 (T16) mutant 2 (T16m2) construct was proven to display the best activity, and it was found to be 4 to 12 times more efficient than nisin, depending on the target organism used. This study reports on the bioengineering and rational design of nisin and tails with activity against Gram-negative bacteria to substantially increase the activity of nisin against MDR Gram-negative pathogens.

RESULTS

**Construction of peptide fusions containing a nisin part and a tail with activity against Gram-negative bacteria.** The first two rings of nisin can bind to lipid II to inhibit cell wall synthesis and serve as a docking point for subsequent pore formation in the membrane (1). However, due to its size, hydrophobicity, and charge, nisin cannot efficiently pass through the outer membrane of Gram-negative bacteria. Fusions containing both the nisin lipid II-binding part (26) and an outer membrane-penetrating part can kill Gram-negative pathogens more successfully than nisin alone due to the Trojan horse effect of the added tail (16). Several new compounds were constructed in which the tail with activity against Gram-negative bacteria was fused to either the A, B, and C rings (ABC rings) of nisin (data not shown), full nisin (Table 2, group 1), or only the A, B, C, D, and E rings (ABCDE rings) of nisin (Table 2, group 2). We found that the

**TABLE 1** Tails fused to a specific part of nisin

Tail	Sequence	Reference	Source
T1	DKPRPYLPRRPV	17	Designed antimicrobial peptide based on statistical analyses
T2	DKYLPRRPV	17	Designed antimicrobial peptide based on statistical analyses
T3	PFKISIH	18	Royal jelly of <i>Apis mellifera</i>
T4	ILPWKWPWWPWR	19	Cytoplasmic granules of bovine neutrophils
T5	ILGKILGKIKLF	20	<i>Opisthacanthus madagascariensis</i>
T6	NGVQPKY	21	White blood cell extracts of Siamese crocodile
T7	NAGSLLSGWG	21	White blood cell extracts of Siamese crocodile
T8	KIAKVALKAL	22	Skin secretions of <i>Xenopus laevis</i>
T9	FLPIAGKLLSGLSGLL	23	Skin secretions of <i>Amolops loloensis</i>
T10	FLPGLLAGLL	24	Skin secretions of <i>Euphyctis cyanophlyctis</i>
T11	AAGMGFFGAR	25	<i>Urechis unicinctus</i>

**TABLE 2** Fusions including the nisin moiety and tails with activity against Gram-negative bacteria

Tail	Group 1		Group 2	
	Name	Sequence	Name	Sequence
T1	T1F	Nisin + DKPRPYLPRRPV	T1S	ABCDE rings + SG + DKPRPYLPRRPV
T2	T2F	Nisin + DKYLPRRPV	T2S	ABCDE rings + SG + DKYLPRRPV
T3	T3F	Nisin + PFKISHL	T3S	ABCDE rings + SG + PFKISHL
T4	T4F	Nisin + ILPWKWPWWPWR	T4S	ABCDE rings + SG + ILPWKWPWWPWR
T5	T5F	Nisin + ILGKILKGKILF	T5S	ABCDE rings + SG + ILGKILKGKILF
T6	T6F	Nisin + NGVQPKY	T6S	ABCDE rings + SG + NGVQPKY
T7	T7F	Nisin + NAGSLLSGWG	T7S	ABCDE rings + SG + NAGSLLSGWG
T8	T8F	Nisin + KIAKVALKAL	T8S	ABCDE rings + SG + KIAKVALKAL
T9	T9F	Nisin + FLPIAGKLLSGLSGLL	T9S	ABCDE rings + SG + FLPIAGKLLSGLSGLL
T10	T10F	Nisin + FLPGLLAGLL	T10S	ABCDE rings + SG + FLPGLLAGLL
T11	T11F	Nisin + AAGMGFFGAR	T11S	ABCDE rings + SG + AAGMGFFGAR

expression of fusions including the ABC rings of nisin and the tails was less efficient than that of the structures in the group 1 and group 2 constructs (data not shown), which was in agreement with previous reports (16). So, we discuss only the fusions with full nisin (group 1) or the five rings of nisin (group 2) in this paper. In order to make the hybrid peptide shorter and more stable, the amino acid sequence IHVSK was deleted in the group 2 mutants. In this case, an SG sequence was added to work as a flexible linker between the ABCDE rings of nisin and the tails (Table 2, group 2). The sequences of the fusions are listed in Table 2. The structures of these fusions are shown in Fig. 1.

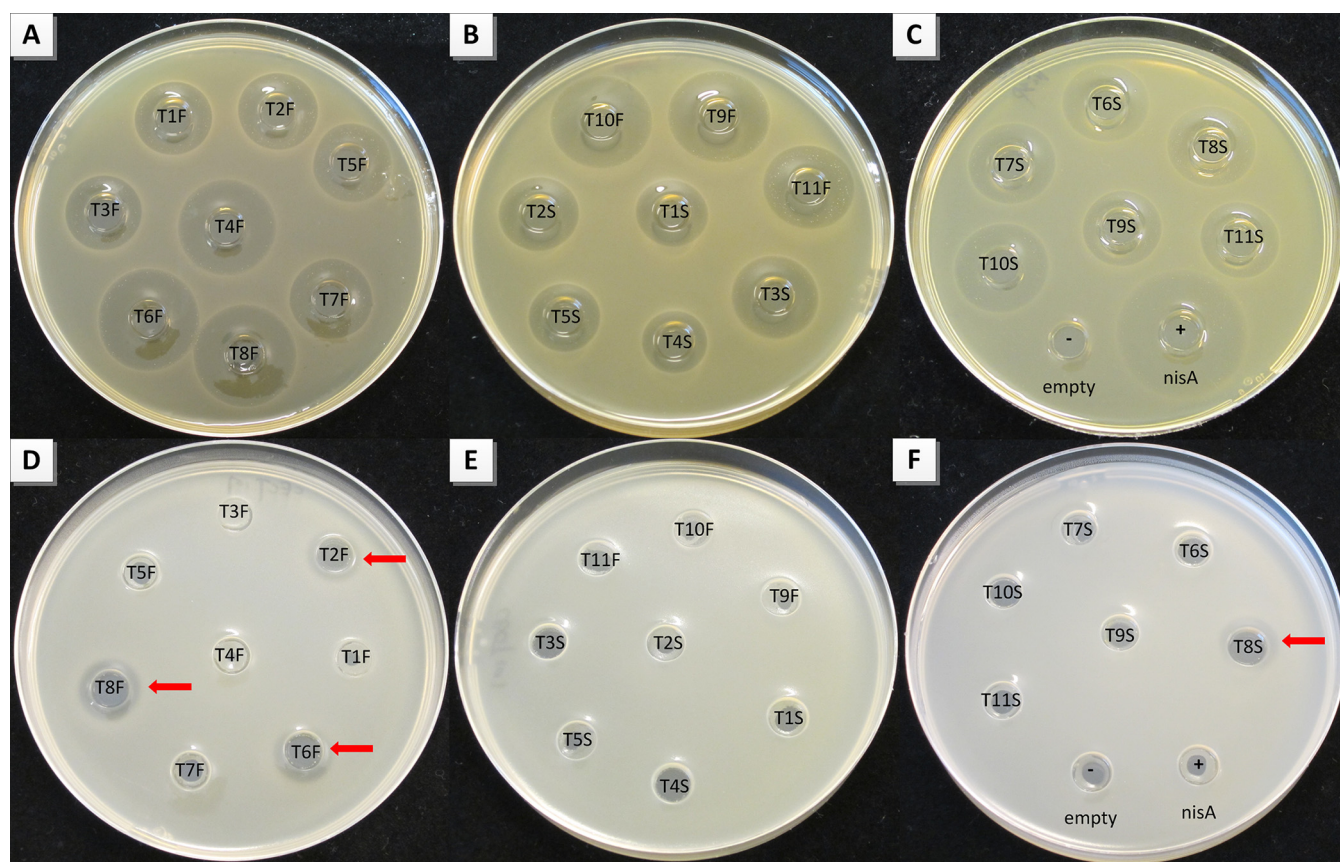
**Characterization of fusions by MS and antimicrobial activity.** All the fusions were induced and expressed with the nisin production system in *L. lactis* and then precipitated with trichloroacetic acid (TCA). Tricine SDS-PAGE was used to check the production level of the hybrid peptides. The mass of the peptides before leader peptide removal was determined via matrix-assisted laser desorption ionization–time of flight MALDI-TOF mass spectrometry (MS) to assess the modification extent of the hybrid peptides. Simultaneously, the antibiotic activity of the peptides activated using either *in situ*-produced (*L. lactis*) or purified (*E. coli*) NisP (27) was tested for qualitative antimicrobial screening. As Table 3 shows, the production level of some fusions was too

**TABLE 3** Results of tricine SDS-PAGE and MS of peptides containing the leader part<sup>a</sup>

Tail	Name	Visible on tricine SDS-polyacrylamide gel	Predicted mass (Da) (no. of dehydrations measured/ no. of possible dehydrations)	Measured mass (Da)	Inferred degradation at:	
					N terminus	C terminus
T1	T1F	–	8,195.66 (9/9)	\	\	\
	T1S	–	7,706.02 (8/8)	\	\	\
T2	T2F	+	5,506.52 (9/9)	5,507.13	ΔMHHHHHHSTKDFNLDL	ΔRPV
	T2S	+	4,897.79 (8/8)	4,898.44	ΔMHHHHHHSTKDFNLDLVSVS	ΔV
T3	T3F	–	7,540.94 (10/10)	\	\	\
	T3S	–	7,051.31 (9/9)	\	\	\
T4	T4F	–	8,512.07 (9/9)	\	\	\
	T4S	–	8,022.43 (8/8)	\	\	\
T5	T5F	–	8,075.71 (9/9)	\	\	\
	T5S	–	7,586.08 (8/8)	\	\	\
T6	T6F	+	7,460.61 (6/9)	7,461.76	No	No
	T6S	+	6,953.30 (6/8)	6,952.85	No	No
T7	T7F	+	7,580.67 (8/11)	7,582.97	No	No
	T7S	+	7,127.35 (5/10)	7,129.11	No	No
T8	T8F	+	6,943.14 (9/9)	6,945.21	ΔMHH	ΔKAL
	T8S	+	5,740.77 (6/8)	5,740.07	ΔMHHHHHHS	ΔLKAL
T9	T9F	–	8,167.76 (11/11)	\	\	\
	T9S	–	7,678.12 (10/10)	\	\	\
T10	T10F	+	7,021.13 (9/9)	7,023.29	ΔM	ΔLAGLL
	T10S	+	6,167.19 (8/8)	6,165.97	ΔMHHHH	ΔGLL
T11	T11F	+	7,603.90 (8/9)	7,601.46	No	No
	T11S	+	7,135.27 (6/8)	7,139.26	No	No

<sup>a</sup>+, visible on tricine SDS-polyacrylamide gel; –, not visible on tricine SDS-polyacrylamide gel; \, no detectable peak in MALDI-TOF MS.





**FIG 2** Screening the hybrid peptides against *L. lactis* and *E. coli* CECT101. Thirty microliters of TCA-precipitated supernatants produced by *L. lactis* NZ9000(pIL3EryBTC/pNZ-mutant) was deposited on the wells. T1F to T11F, group 1 hybrid peptides containing full nisin fused to tails with activity against Gram-negative bacteria; T1S to T11S, group 2 hybrid peptides containing ABCDE rings of nisin and the hinge region (serine and glycine) fused to tails with activity against Gram-negative bacteria; nisA, the positive control, consisting of a TCA-precipitated supernatant of NZ9000(pIL3EryBTC/pNZ-nisA); empty, negative control, consisting of the precipitated supernatant of NZ9000(pIL3EryBTC/pNZ8048). The indicator strains were *L. lactis* NZ9000(pNZnisP8H) (A to C) and *E. coli* CECT101 (D to F).

low to show a clearly visible band in tricine SDS-PAGE, while some others were produced with high yields comparable to the yield of wild-type nisin produced using the same system (see Fig. S1 in the supplemental material). The mass results indicate that the peptides T6F/S, T7F/S, and T11F/S were not fully dehydrated. In all cases, a minimum of 5 dehydrations was detected. Considering the N-to-C directionality of NisBC, 5 dehydrations are enough to correctly construct ABC rings which preserve the lipid II-binding capacity of nisin and retain partial antimicrobial activity (26, 28). We observed that the mutant peptides T2F/S, T10F/S, and T8F/S rendered a peptide with a mass smaller than the predicted mass. This might be due to degradation during production or TCA precipitation. The nonspecific cleavage site in these peptides was not dependent on the sequence of the tail since the mass difference between fusions to full nisin and fusions to the ABCDE rings was not conserved (i.e., the full nisin and group 2 fusions were truncated differently). This phenomenon has been previously observed with fusions between nisin and tails with activity against Gram-negative bacteria (16). All of them exerted relatively high activity against *L. lactis* NZ9000(pNZnisP8H), but only T2F, T6F, T8F, and T8S showed activity against *E. coli* CECT101 (Fig. 2). This was probably observed because the first two rings of nisin can bind to lipid II, while the last two rings can participate in pore formation, leading to the death of *L. lactis* (26, 27). It could also be partly due to the sensitivity of *L. lactis* NZ9000(pNZnisP8H), which was selected in the first screening for its reported increased sensitivity (27). Thus, even poorly active or poorly produced compounds with activity

**TABLE 4** Mutants for T6, T8, and GNT16

Name	Sequence
T6m1	ABCDE rings + SG + NGVQPKYK
T6m2	ABCDE rings + SV + NGVQPKYK
T6m3	ABCDE rings + SV + NGVQPKY
T6m4	ABC rings + NGVQPKY + DE rings + SIHVSK
T8m1	ABCDE rings + SG + KIAKVALKALK
T8m2	ABCDE rings + SV + KIAKVALKALK
T8m3	ABCDE rings + SV + KIAKVALKAL
T8m4	ABC rings + KIAKVALKAL + DE rings + SIHVSK
T16m1	ABCDE rings + SG + PRPPHPRLK
T16m2	ABCDE rings + SV + PRPPHPRLK
T16m3	ABCDE rings + SV + PRPPHPRL
T16m4	ABC rings + PRPPHPRL + DE rings + SIHVSK

against *L. lactis* NZ9000(pNZnisP8H) that would otherwise have been discarded during a screening against solely *E. coli* could be detected.

**Designing mutants of tail 6, tail 8, and GNT16.** Fusions containing tails 2, 6, and 8 showed a good potential for further tests for their activity against Gram-negative pathogens. In addition, GNT16 has been reported by our group before to have relatively good trans-outer membrane activity (16). Both tail 2 (DKYLPRPRPV) and tail 16 (PRPPHPRL) are proline-rich peptides, like GNT16. So, tails 6, 8, and 16 were selected to be rationally modified and further studied, as shown in Table 4. For TXm1 (where X is tail 6, 8, or 16), a hydrophilic and positively charged lysine was added at the C terminus of the peptides TXS (where X is tail 6, 8, or 16), because many lantibiotics have a lysine at the C terminus. In the mutants TXm2 (where X is tail 6, 8, or 16), the glycine in the linker region between the nisin moiety and the tail in TXm1 was replaced by valine, since valine can make this region a bit less flexible. TXm3 (where X is tail 6, 8, or 16) mutants were composed of five rings of nisin and tails with SV between. TXm4 (where X is tail 6, 8, or 16) mutants were designed in a totally different manner, using the peptide tail sequences with activity against Gram-negative bacteria as the hinge region between the ABC rings and DE rings of nisin, thus replacing the original NMK in the hinge region (Table 4).

**Characterization of purified leaderless peptides.** The active peptides were acquired by purification and digestion using purified NisP (27) to remove the leader part and activate the peptide. Their masses were measured by MALDI-TOF MS (Fig. S2), and the mass results are listed in Table 5, which also shows the yields of the peptides. The results show that the peptides with tails 6 and 16 were not fully dehydrated, while peptides containing tail 8 were most likely degraded during the process of production or purification by exoproteases. T8m4, where the tail was placed as a hinge region and therefore not accessible to exoproteases, stayed as a full-length peptide, and no degradation was observed. The alkylation reaction with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) proved that there were no free thiol groups in the peptides (Fig. S3). This fact, together with the activity, strongly suggests that the lanthionine rings possess the right regiochemistry.

**Activity of the constructed fusions against Gram-negative pathogens.** Tests of the activity of the fusion mutants of T6, T8, and T16 against five Gram-negative pathogens were performed. The results of the MIC tests are provided in Table 6. It is clear that the potency of nisin against these Gram-negative pathogens is reduced in comparison to the activity at nanomolar concentrations that it displays against *L. lactis*. The MIC value of the designed peptides ranged from 6  $\mu$ M, needed to inhibit *A. baumannii*, to more than 48  $\mu$ M, needed to inhibit *K. pneumoniae*. The activity of most of the mutants slightly outperformed that of nisin against some of the strains tested. A noteworthy finding was that T6F had improved activity over that of nisin against five out of the six strains tested, including *K. pneumoniae*.

After further design, TXm2 (where X is tail 6, 8, or 16) had lower MIC values than TXm1, TXm3, TXm4, and, especially, T16m2. T16m2 displayed 4 to 12 times better

**TABLE 5** MS analysis and yields of leaderless peptides used for activity test<sup>a</sup>

Name	Predicted mass (Da) (no. of dehydrations measured/no. of possible dehydrations)	Measured mass (Da)	Inferred degradation	Yield (μg/liter)
T6F	4,160.07 (7/9)	4,158.66	No	834
T6S	3,652.44 (7/8)	3,654.34	No	940
T6m1	3,780.61 (7/8)	3,778.06	No	1,218
T6m2	3,822.69 (7/8)	3,822.82	No	1,138
T6m3	3,676.52 (8/8)	3,678.67	No	740
T6m4	3,768.60 (8/9)	3,766.75	No	1,460
T8F	4,186.14 (9/9)	4,186.03	ΔAL	740
T8S	3,324.08 (6/8)	3,322.60	ΔVALKAL	640
T8m1	3,696.83 (8/8)	3,695.53	ΔALK	246
T8m2	3,777.70 (6/8)	3,777.98	ΔALK	299
T8m3	3,366.16 (6/8)	3,366.81	ΔVALKAL	260
T8m4	4,035.99 (7/9)	4,033.09	No	414
GNT16	4,324.32 (7/9)	4,326.29	No	340
GNT16SG	3,816.69 (7/8)	3,815.64	No	264
T16m1	3,944.87 (7/8)	3,941.44	No	234
T16m2	3,966.00 (8/8)	3,966.68	No	498
T16m3	3,855.90 (7/8)	3,854.75	No	316
T16m4	3,932.85 (8/9)	3,930.71	No	483
Nisin	3,352.61 (8/9)	3,354.75	No	ND <sup>b</sup>

<sup>a</sup>Nisin was purified from a commercial 2.5% preparation.<sup>b</sup>ND, not determined.

activity than nisin against all the Gram-negative pathogens used, including *Enterobacter aerogenes* and *K. pneumoniae*, which were the bacteria most resistant to these compounds under the conditions tested. Of note, T16m2 was 12 times more active than nisin against *K. pneumoniae* (a  $\beta$ -lactamase-producing strain) and *A. baumannii*.

On the other hand, among the mutants, TXm4 (where X is tail 6, 8, or 16) showed the worst activity against Gram-negative pathogens. None of these peptides displayed improved activity but, rather, displayed a drastic increase in the MIC value.

**TABLE 6** MICs of nisin, fusion peptides, and tails<sup>a</sup>

	<i>E. coli</i> LMG15862	<i>K. pneumoniae</i> LMG20218	<i>P. aeruginosa</i> LMG 6395	<i>A. baumannii</i> LMG01041	<i>E. aerogenes</i> LMG02094	<i>L. lactis</i> MG1363
Nisin	12	48	36	6	32	0.006
T6F	8	32	16	2	64	ND
T6S	32	>64	64	6	64	ND
T6m1	32	>32	32	4	64	ND
T6m2	8	32	32	2	16	0.375
T6m3	32	>64	>64	16	>64	ND
T6m4	64	>64	>64	8	>64	0.25
T8F	32	>64	32	3	>64	ND
T8S	16	64	32	3	64	ND
T8m1	16	>64	32	2	64	ND
T8m2	4	8	8	2	16	0.3
T8m3	16	32	32	4	64	ND
T8m4	>64	>64	>64	64	>64	0.2
GNT16	6	12	12	4	12	ND
GNT16SG	>32	>64	>32	8	>32	ND
T16m1	4	16	16	1	16	ND
T16m2	2	4	8	0.5	4	0.25
T16m3	8	16	>64	1	16	ND
T16m4	64	>64	>64	8	>64	0.5
Tail T6m2	>256	>256	>256	>256	>256	>32
Tail T8m2	>256	>256	>256	>256	>256	>32
Tail T16m2	>256	>256	>256	>256	>256	>32

<sup>a</sup>MICs are in micromolars. Green, nisin and MIC values of nisin; red, MIC values which were lower than those of nisin; ND, not determined. The data in the last three rows represent the activity of the synthetic added tails alone, the sequences of which were as follows: NGVQPKYK for Tail T6m2, KIAKVALKALK for Tail T8m2, and PRPPHPLK for Tail T16m2.



**TABLE 7** MIC of nisin against Gram-negative pathogens in the presence of EDTA

Concn of EDTA ( $\mu$ M)	MIC of nisin ( $\mu$ M)				
	<i>E. coli</i> LMG15862	<i>K. pneumoniae</i> LMG20218	<i>P. aeruginosa</i> LMG6395	<i>A. baumannii</i> LMG01041	<i>E. aerogenes</i> LMG02094
0	12	48	36	6	32
50	6	48	18	6	32
100	3	36	9	3	8
200	3	24	2.25	1.5	8
400	1.5	12	0.6	0.75	4

**Unraveling the effect of the fusions on nisin activity.** The added tails for the best three candidates, T6m2, T8m2 and T16m2, were synthesized as small peptides and tested for their activity against Gram-negative pathogens and the Gram-positive bacterium *L. lactis* MG1363. The MIC values are listed in Table 6. None of the three tails alone showed activity against Gram-negative pathogens at the concentrations tested, with the MIC values being higher than 256  $\mu$ M. Similarly, none of these three tail peptides could inhibit the growth of *L. lactis* at a concentration of 32  $\mu$ M. These results indicate that the main role of the added tails was to assist the nisin part to pass through the outer membrane, thus working as a carrier or gate opener.

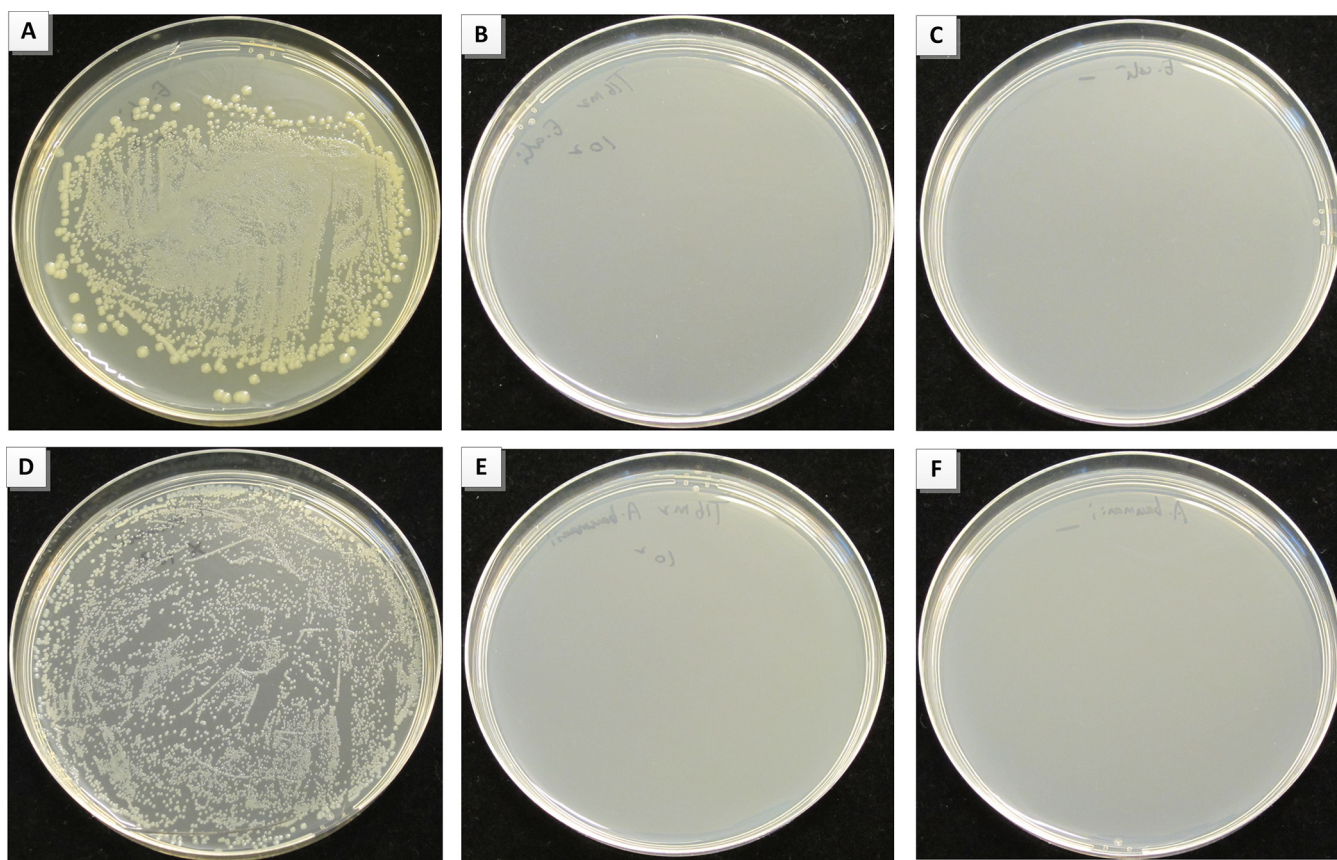
Next we investigated the effect of the added tails on the intrinsic killing mechanism of nisin. We used the Gram-positive bacterial species *L. lactis* as a model organism and confronted it with TXm2 and TXm4 mutants, where the tail behaves either as a C-terminal addition or as a hinge region, respectively. The MIC values of nisin and 6 fusions, i.e., T6m2, T8m2, T16m2, T6m4, T8m4, and T16m4, are listed in Table 6. The MIC values of these 6 peptides were 62 times, 50 times, 42 times, 42 times, 33 times, and 83 times higher than the MIC of nisin, respectively. This clearly indicates that the tails have a negative influence on the intrinsic activity of nisin on the cytoplasmic membrane.

**Effect of EDTA on the activity of nisin against Gram-negative pathogens.** As reported before (13), when sufficient EDTA is added, a smaller amount of nisin is needed to kill Gram-negative bacteria. In order to assess if the fusions changed the spectrum of activity of nisin, we decided to compare the MIC of nisin in the presence of EDTA with that of the fusion peptides alone. In this work, different concentrations of EDTA were added together with nisin to test their activity against Gram-negative pathogens. Untreated cells were used as a positive control. The results are listed in Table 7. We could see that the pathogens displayed a different sensitivity to nisin when EDTA was used as an adjuvant. When 50  $\mu$ M EDTA was added, the MIC value of nisin against *E. coli* and *P. aeruginosa* decreased 2 times, while the MIC value against *K. pneumoniae*, *A. baumannii* and *E. aerogenes* did not change. At least 100  $\mu$ M EDTA was needed to reduce the MIC value more than 2-fold, with the MIC for *K. pneumoniae* being an exception. When comparing these MIC data (Table 7) and those for the nisin fusions alone (Table 6), it was very obvious that the spectrum of activity of nisin was changed in the presence of either EDTA or the added tails.

**Bactericidal effect of T16m2.** The bactericidal effect of T16m2, the best candidate in our hands, was determined. Unlike the positive controls (untreated cells), no colonies of *E. coli* or *A. baumannii* could grow after incubation with T16m2 (10-fold MIC) overnight (Fig. 3). There was no growth recovery after exposure to T16m2, so T16m2 was proven to be bactericidal.

## DISCUSSION

Antimicrobial resistance has become an imminent and ever-increasing global problem which threatens public health and economic development. It has been reported that hospital infections caused by *P. aeruginosa* and *A. baumannii* are difficult to treat, since these pathogens are often resistant to most of the drugs used clinically (29). What is more, *E. aerogenes* and *K. pneumoniae* strains resistant to carbapenem and cepha-



**FIG 3** Determination of viable cells after treatment with fusion peptides. (A) Positive control for *E. coli*, consisting of 50  $\mu$ l of a 100-fold-diluted sample from the well with *E. coli* alone; (B) 50  $\mu$ l of a diluted sample from the well in which *E. coli* was treated with T16m2; (C) medium control (no bacteria were inoculated in this well); (D) positive control for *A. baumannii*, consisting of 50  $\mu$ l of a 100-fold-diluted sample from the well with *A. baumannii* alone; (E) 50  $\mu$ l of a 100-fold-diluted sample from the well in which *A. baumannii* was treated with T16m2; (F) medium control (no bacteria were inoculated in this well).

losporins have already been isolated (29, 30). There are pressing and urgent demands for the discovery of new antimicrobials to act against Gram-negative pathogens. Various authors indicate that antimicrobial peptides can constitute a suitable source of novel compounds with activity against Gram-negative bacteria. In this work, we investigated rationally designed nisin mutants for this purpose.

Nisin is the best-studied lantibiotic and exhibits high activity against Gram-positive bacteria, while its activity against Gram-negative microorganisms is drastically reduced. The outer membrane of Gram-negative bacteria acts as a good protective barrier for nisin to pass through and thereby prevents nisin from reaching the inner membrane. In the presence of chelating agents or sublethal outer membrane perturbation, nisin can inhibit the growth of Gram-negative bacteria. However, the chelators or stress is not appropriate for most applications. In order to increase the ability of nisin to reach the inner membrane, we performed extensive engineering in this work. We chose a set of peptides that can naturally target Gram-negative bacteria and designed nisin fusions with tails that work as a Trojan horse. Their activities against five clinically relevant (drug-resistant) Gram-negative bacterial species were assayed. We created functional fusions between nisin, which combines with the pyrophosphate in lipid II (an under-exploited drug target), and several peptides with the capacity to cross the outer membrane of Gram-negative bacteria.

The peptides with activity against Gram-negative bacteria were selected primarily on the basis of their capability of outer membrane penetration and their inclusion in different structural groups (proline-rich, arginine-rich, or cationic peptides). The preliminary screening and previous data focused our interest on tails 6, 8, and 16 fused to

the ABCDE rings of nisin. We confirmed that adding a tail with activity against Gram-negative bacteria to either nisin or a truncated version of nisin is an efficient way to improve the activity of nisin against Gram-negative pathogens. However, the addition of an extra stretch of amino acids can make it prone to partial proteolytic degradation, as we could observe for the T2, T8, and T10 mutants (Table 3). These mutants rendered one major product that had a reduced mass but that still retained activity.

The set of mutants where the tail with activity against Gram-negative bacteria replaced nisin's hinge region, TXm4 (where X is tail 6, 8, or 16), failed to show activity against Gram-negative pathogens in this study. However, they still retained activity against *L. lactis* MG1363 (Table 6). Thus, the outer membrane-penetrating capacity of the TXm4 mutants was, in the best case, minimal, which indicates that the tails used in our study could optimally perform the expected activity when they were used as C-terminal extensions of nisin. Moreover, the results confirmed that changes in the hinge region affect the structure and activity of the entire peptide (31). The location of the tail in the hinge region in TXm4 decreased its outer membrane-traversing capacity and, therefore, that of the whole fusion peptide. Our results clearly discourage the use of the selected tails as a replacement of the nisin hinge region for antimicrobial activity improvement.

Variations in the region linking ring E of nisin to the peptide with activity against Gram-negative bacteria (the Gly-to-Val mutation in T8m3 and T16m3) exerted better activity against specific pathogens than T8S and GNT16SG, respectively. T8m3 was 2 times more active than T8S against *K. pneumoniae*, while T16m3 was more than 4 times more active than GNT16SG. T16m3 was remarkably more active than GNT16SG against *E. coli* (MICs, 8  $\mu$ M and >32  $\mu$ M, respectively), *K. pneumoniae* (MICs, 16  $\mu$ M and >64  $\mu$ M, respectively), *A. baumannii* (MICs, 1  $\mu$ M and 8  $\mu$ M, respectively), and *E. aerogenes* (MICs, 16  $\mu$ M and >32  $\mu$ M, respectively). These results establish that replacing glycine with valine in the linker region significantly enhances the activity of these peptides against the tested Gram-negative pathogens.

Since most lantibiotics described previously in the literature either contain a positively charged amino acid at the C terminus or have undergone specific enzymatic C-terminal decarboxylation, a set of mutants with an extra lysine was created to mimic that situation. T8m2 was 2 to 6 times more active than nisin against selected pathogenic strains. The activity of T16m2 against *P. aeruginosa*, *E. coli*, and *E. aerogenes* was enhanced 4.5 times, 6 times, and 8 times, respectively. Most notably, the MIC value of T16m2 against *K. pneumoniae* and *A. baumannii* was 12 times lower than that of nisin.

T16m1, T16m2, and T16m3 shared the same tail with activity against Gram-negative bacteria with GNT16 and GNT16SG, but T16m2 rendered the best results, followed by T8m2. The difference in the MIC value of T16m2 from that of GNT16SG against *E. coli* and *K. pneumoniae* was more than 16-fold. This finding indicates the importance of a C-terminal lysine as well as valine in the flexible linker region connecting nisin and the tail. The reason might be that the lysine residue at the C terminus has a better interaction with the negatively charged phospholipids to facilitate translocation, while a valine in the linker region might facilitate interaction with the membrane lipophilic part. However, the effect of the Gly-to-Val mutation was detrimental for the activity of T16m3. This effect was counteracted when lysine was added in T16m2, which outperformed mutant T16m1, where glycine is present. Collectively, our results show that the simultaneous mutation of glycine to valine and the insertion of a C-terminal lysine improved the antimicrobial activity of all the constructs.

T6m2, T8m2, and T16m2 were proven to be the best candidates in their specific similar tail sets. As shown in Table 6, the low levels of activity of the tails of T6m2, T8m2, and T16m2 alone against Gram-negative pathogens (MIC value > 256  $\mu$ M) and the Gram-positive bacterium *L. lactis* MG1363 (MIC value > 32  $\mu$ M) illustrates the role of the tails as mainly transmembrane carriers rather than as bactericidal agents themselves. The nisin-tail fusions were more than 50 times less potent against *L. lactis* MG1363 than nisin. Thus, the activity of the nisin part on the inner membrane was extremely



compromised by addition of the tails. After treatment with different concentrations of EDTA, a smaller amount of nisin was needed to kill the Gram-negative pathogens. However, the spectrum of activity of the nisin fusions against Gram-negative pathogens was different from that of nisin against Gram-negative pathogens that had been treated with EDTA. T16m2 was shown to be 6 times, 12 times, 4.5 times, 12 times, and 8 times more active than nisin against *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *A. baumannii*, and *E. aerogenes*, respectively. For comparison, these pathogens treated with 200  $\mu$ M EDTA were 4 times, 2 times, 16 times, 4 times, and 4 times more sensitive to nisin, respectively (Table 7). We also showed that T16m2 exerts a bactericidal effect against *E. coli* and *A. baumannii* (Fig. 3). In conclusion, the tails of the fusions changed both the activity and the spectrum of activity of nisin, but the fusion was still bactericidal.

Previous work (16) showed that the activity of the model lantibiotic nisin against *E. coli* can be improved 2-fold by combination of the functional domains of different antimicrobial peptides, namely, apidaecin and nisin. Our data show that by rational design it is possible to further improve the activity of nisin up to 12-fold against selected pathogenic (drug-resistant) Gram-negative bacteria either in healthcare or in food. These data provide new design principles for further engineering that can lead to the development of highly potent lantibiotic derivatives specifically targeting Gram-negative bacteria. Applications could range from food protection to clinical applications. For the latter, further preclinical studies on toxicity, stability, hemolysis, and pharmacokinetics/pharmacodynamics would be required.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The bacteria used in this study are listed in Table 8. *L. lactis* strains were cultured in M17 broth supplemented with 0.5% (wt/vol) glucose (GM17) or GM17 agar for genetic manipulation or in minimal expression medium (MEM) (3) for protein expression at 30°C.

*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Enterobacter aerogenes* were grown in shaken Luria-Bertani (LB) broth or on LB agar at 37°C. The bacterial strains with the prefix LMG were obtained from the Belgian Coordinated Collections of Microorganisms (BCCM).

Chloramphenicol and/or erythromycin was used at 5  $\mu$ g/ml when necessary.

**Molecular cloning.** Molecular cloning techniques were performed as described by Sambrook and Russell (35). Preparation of competent cells and transformation were performed as described by Holo and Nes (36). Restriction enzymes and ligase were supplied by Thermo Fisher.

**Construction of expression vectors.** The peptidic tails were added to nisin genetically by round PCR. The primers (Table 9) were designed to insert the tails between the nisin part and the restriction site HindIII. Each pair of primers contained a part annealing with the template vector pNZnisA leader His2 and a part encoding the peptide tail.

After amplification, the PCR cleanup products were digested using DpnI to digest the template and ligated overnight. The ligation product was desalted and transformed into strain NZ9000, isolated, extracted, and sequenced to verify the integrity of the sequence.

**Protein expression.** Each vector containing the mutant structural gene was transformed into NZ9000(pIL3EryBTC). Cells were first cultured overnight in GM17 medium with 5  $\mu$ g/ml chloramphenicol and 5  $\mu$ g/ml erythromycin and transferred into MEM (3) at a final concentration of 2%. Nisin (5 ng/ml) was added at the beginning of the inoculation and when the culture reached an optical density at 600 nm (OD<sub>600</sub>) of 0.4 to 0.6. Cells were harvested 3 h after the second induction by centrifugation for 20 min at 6,500 rpm at 4°C. The supernatant was kept for purification.

**Protein purification, characterization, and quantification.** For fast detection of the designed peptides, a small volume of culture supernatant was used for precipitation using trichloroacetic acid (TCA) as described by Sambrook and Russell (35), and the concentrated peptides were loaded on a 16% tricine SDS-polyacrylamide gel (37). Alternatively, for larger-volume ( $\geq 1$ -liter) cultures, the peptide was concentrated by cationic exchange chromatography and gel filtration (28). Samples were freeze-dried afterwards.

The freeze-dried sample was dissolved in 50 mM ammonium acetate, pH 6.0, for overnight digestion with purified NisP (27). The active peptide was further purified by high-performance liquid chromatography (HPLC) as indicated elsewhere (31). The fractions were collected, tested for activity against *L. lactis*, and analyzed by MALDI-TOF MS (34). The active and pure fraction was lyophilized and stored as a powder until further use.

The quantification was performed by HPLC, as described previously (34). The synthetic peptides T6m2, T8m2, and T16m2 were synthesized and provided with  $>99\%$  purity by Proteogenix (France).

Nisin was purified from commercial 2.5% powder as described by Slootweg et al. (38).

**Free-thiol alkylation.** To investigate whether the fusions possessed free cysteine residues, reactions with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) were performed. A linear peptide, ADP (H-GIGKHVGKALKGLKLLKGLGEC-OH), was used as a control. The reaction with CDAP was per-

**TABLE 8** Strains and plasmids used in this work<sup>a</sup>

Strain or plasmid	Characteristic	Purpose	Reference or source
<b>Strains</b>			
<i>Lactococcus lactis</i> NZ9000	<i>nisRK</i>	Expression host and indicator strain	32
<i>Lactococcus lactis</i> NZ9000(pNZnisP8H)	<i>nisP</i>	Indicator strain	27
<i>Lactococcus lactis</i> MG1363		Indicator strain	33
<i>Escherichia coli</i> CECT101		Indicator strain	Lab collection
<i>Escherichia coli</i> LMG15862	$\beta$ -Lactamase	Indicator strain	Lab collection (BCCM)
<i>Klebsiella pneumoniae</i> LMG20218	$\beta$ -Lactamase	Indicator strain	Lab collection (BCCM)
<i>Pseudomonas aeruginosa</i> LMG6395		Indicator strain	Lab collection (BCCM)
<i>Acinetobacter baumannii</i> LMG01041		Indicator strain	Lab collection (BCCM)
<i>Enterobacter aerogenes</i> LMG02094		Indicator strain	Lab collection (BCCM)
<b>Plasmids</b>			
pNZnisA leader his2	Cm <sup>r</sup> <i>nisA</i> , encoding nisin, with 6 His residues inserted behind the first methionine	Expression vector, expression with a 6-His-tagged nisin	16
pNZ8048	Cm <sup>r</sup>	Expression vector, used as the negative control in activity test	7
pIL3EryBTC	Ery <sup>r</sup> <i>nisBTC</i> , under the control of P <sub>nisA</sub>	Modification and transport of lantibiotics	34
pNZnisA T6	Cm <sup>r</sup> <i>nisA</i> , T6	Expression of nisin with a 6-His tag and a NGVQPKY tail	This work
pNZnisA T6S	Cm <sup>r</sup> <i>nisA</i> ( $\Delta$ 30–34), GNGVQPKY	Expression of hybrid peptide with a 6-His tag	This work
pNZnisA T8	Cm <sup>r</sup> <i>nisA</i> , T8	Expression of nisin with a 6-His tag and a KIAKVALKAL tail	This work
pNZnisA T8S	Cm <sup>r</sup> <i>nisA</i> ( $\Delta$ 30–34), GKIAKVALKAL	Expression of a hybrid peptide with a His tag	This work
pNZnisA GNT16	Cm <sup>r</sup> <i>nisA</i> , GNT16	Expression of nisin with a 6-His tag and a PRPPHPRL tail	16
pNZnisA GNT16S	Cm <sup>r</sup> <i>nisA</i> ( $\Delta$ 30–34), GPRPPHPRL	Expression of a hybrid peptide with a 6-His tag	16
pNZnisA Ts	Cm <sup>r</sup> <i>nisA</i> , Ts	Expression of nisin or nisin( $\Delta$ 30–34) with a 6-His tag and the tails listed in Table 2	This work
pNZnisA T6m1 to pNZnisA T6m3	Cm <sup>r</sup> <i>nisA</i> , T6 mutants	Expression of a hybrid peptide containing nisin( $\Delta$ 30–34) with a 6-His tag and the tails listed in Table 4	This work
pNZnisA T6m4	Cm <sup>r</sup> , T6 as the hinge region between the ABC rings and the DE rings of nisin	Expression of a hybrid peptide	This work
pNZnisA T8 m1 to pNZnisA T8 m3	Cm <sup>r</sup> <i>nisA</i> , T8 mutants	Expression of a hybrid peptide containing nisin( $\Delta$ 30–34) with a 6-His tag and the tails listed in Table 4	This work
pNZnisA T8m4	Cm <sup>r</sup> , T8 as the hinge region between the ABC rings and the DE rings of nisin	Expression of a hybrid peptide	This work
pNZnisA T16 m1 to pNZnisA T16 m13	Cm <sup>r</sup> <i>nisA</i> , T16 mutants	Expression of a hybrid peptide containing nisin( $\Delta$ 30–34) with a 6-His tag and the tails listed in Table 4	This work
pNZnisA T16m4	Cm <sup>r</sup> , T16 as the hinge region between the ABC rings and the DE rings of nisin	Expression of a hybrid peptide	This work

<sup>a</sup>Cm<sup>r</sup>, chloramphenicol resistance; Ery<sup>r</sup>, erythromycin resistance; *nisA*( $\Delta$ 30–34), deletion of gene coding IHVSK in *nisA*; and nisin( $\Delta$ 30–34), deletion of IHVSK in nisin.

formed as described previously (39). The mass spectra before and after the reaction were recorded via MALDI-TOF MS (34).

**Determination of antimicrobial activity and MIC.** Antimicrobial activity was performed by a well diffusion assay as described previously (34). MIC tests were performed in triplicate by liquid growth inhibition microdilution assays according to standard methods at 37°C overnight (40). Growth inhibition was assessed by measuring the OD<sub>600</sub> using a microplate reader (Tecan Infinite F200). The lowest concentration of the antimicrobials that inhibited detectable growth of the indicator strain was identified as the MIC.



**TABLE 9** Primers for PCRs used in this study

Mutant	Primer	Nucleotide sequence
T1F	T1 Fwd	TCC ATA CCT TCC ACG TCC ACG TCC AGT TTA AGC TTT CTT TGA ACC AAA ATT AG
	T1F Rev	GTG GAA GGT ATG GAC GTG GTT TAT C TT TGC TTA CGT GAA TAC TAC AAT G
T1S	T1S Rev	GTG GAA GGT ATG GAC GTG GTT TAT CAC CAC TAC AAT GAC AAG TTG CTG
T2F	T2 Fwd	AAA TAC CTT CCA CGT CCA CGT CCA GTT TAA GCT TTC TTT GAA CCA AAA TTA G
	T2F Rev	CGT GGA CGT GGA AGG TAT TTA TC T TTG CTT ACG TGA ATA CTA CAA TG
T2S	T2S Rev	TGG ACG TGG AAG GTA TTT ATC ACC ACT ACA ATG ACA AGT TGC T
T3F	T3 Fwd	AAA ATC TCA ATC CAC CTT TAA GCT TTC TTT GAA CCA AAA TTA G
	T3F Rev	TAA AGG TGG ATT GAG ATT TTG AAT GGT TTG CTT ACG TGA ATA CTA C
T3S	T3S Rev	GGT GGA TTG AGA TTT TGA ATG GAC CAC TAC AAT GAC AAG TTG
T4F	T4 Fwd	GCC ATG GTG GCC ATG GCG TCG TTA AGC TTT CTT TGA ACC AAA ATT AG
	T4F Rev	GGC CAC CAT GGC CAT TTC CAT GGA AGG AT T TTG CTT ACG TGA ATA CTA CAA T
T4S	T4S Rev	GGC CAC CAT GGC CAT TTC CAT GGA AGG ATA CCA CTA CAA TGA CAA GTT G
T5F	T5 Fwd	AAG GTA TCA AAA AAC TTT TCT AAG CTT TCT TTG AAC CAA AAT TAG
	T5F Rev	TTG ATA CCT TTA AGG ATT TTA CCA AGG ATT TTG CTT ACG TGA ATA CTA C
T5S	T5S Rev	TTG ATA CCT TTA AGG ATT TTA CCA AGG ATA CCA CTA CAA TGA CAA GTT G
T6F	T6 Fwd	GGT GTT CAA CCA AAA TAC TAA GCT TTC TTT GAA CC
	T6F Rev	ATT TTG GTT GAA CAC CGT TTT TGC TTA CGT GAA TAC TAC
T6S	T6S Rev	ATT TTG GTT GAA CAC CGT TAC CAC TAC AAT GAC AAG TTG
T7F	T7 Fwd	TCA CTT CTT TCA GGT TGG GGT TAA GCT TTC TTT GAA CCA AAA TTA G
	T7F Rev	CCA ACC TGA AAG AAG TGA ACC AGC GTT TTT GCT TAC GTG AAT ACT AC
T7S	T7S Rev	AAC CTG AAA GAA GTG AAC CAG CGT TAC CAC TAC AAT GAC AAG TTG C
T8F	T8 Fwd	AAG TTG CTC TTA AAG CTC TTT AAG CTT TCT TTG AAC CAA
	T8F Rev	CTT TAA GAG CAA CTT TAG CGA TTT TTT TGC TTA CGT GAA TAC TAC AAT G
T8S	T8S Rev	CTT TAA GAG CAA CTT TAG CGA TTT TAC CAC TAC AAT GAC AAG TTG
T9F	T9 Fwd	ACT TCT TTC AGG TCT TTC AGG TCT TCT TTA AGC TTT CTT TGA ACC AAA ATT AG
	T9F Rev	GAA AGA CCT GAA AGA AGT TTA CCA GCG ATT GGA AGG AAT TTG CTT ACG TGA ATA CTA C
T9S	T9S Rev	GAA AGA CCT GAA AGA AGT TTA CCA GCG ATT GGA AGG AAA CCA CTA CAA TGA CAA GTT G
T10F	T10 Fwd	CAG GTC TTC TTG CTG GTC TTC TTT AAG CTT TCT TTG AAC CAA AAT TAG
	T10F Rev	CAG CAA GAA GAC CTG GAA GGA ATT TGC TTA CGT GAA TAC TAC AAT G
T10S	T10S Rev	CAG CAA GAA GAC CTG GAA GGA AAC CAC TAC AAT GAC AAG TTG
T11F	T11 Fwd	TGG GTT TCT TCG GTG CTC GTT AAG CTT TCT TTG AAC CAA AAT TAG
	T11F Rev	GCA CCG AAG AAA CCC ATA CCA GCA GCT TTG CTT ACG TGA ATA CTA C
T11S	T11S Rev	GCA CCG AAG AAA CCC ATA CCA GCA GCA CCA CTA CAA TGA CAA GTT GC
T6m1	T6m1 Fwd	AAC GGT GTT CAA CCA AAA TAC AAG TAA GCT TTC TTT GAA CC
	T6S Rev	ATT TTG GTT GAA CAC CGT TAC CAC TAC AAT GAC AAG TTG
T6m2	T6m1 Fwd	AAC GGT GTT CAA CCA AAA TAC AAG TAA GCT TTC TTT GAA CC
	T6m2 Rev	GTA TTT TGG TTG AAC ACC GTT TAC ACT ACA ATG ACA AGT TGC TG
T6m3	T6F Fwd	GGT GTT CAA CCA AAA TAC TAA GCT TTC TTT GAA CC
	T6m2 Rev	GTA TTT TGG TTG AAC ACC GTT TAC ACT ACA ATG ACA AGT TGC TG
T6m4	T6m4 Fwd	CGG TGT TCA ACC AAA ATA CAC AGC AAC TTG TCA TTG TAG
	T6m4 Rev	GTA TTT TGG TTG AAC ACC GTT ACA ACC CAT CAG AGC TCC TG
T8m1	T8m1 Fwd	CGC TAA GGT TGC TCT TAA AGC TCT TAA GTA AGC TTT CTT TGA AC
	T8m1 Rev	GAG CTT TAA GAG CAA CCT TAG CGA TTT TAC CAC TAC AAT GAC AAG TTG
T8m2	T8m1 Fwd	CGC TAA GGT TGC TCT TAA AGC TCT TAA GTA AGC TTT CTT TGA AC
	T8m2 Rev	GAG CTT TAA GAG CAA CCT TAG CGA TTT TTA CAC TAC AAT GAC AAG TTG CTG TTT TC
T8m3	T8m3 Fwd	CGC TAA GGT TGC TCT TAA AGC TCT TTA AGC TTT CTT TGA ACC
	T8m2 Rev	GAG CTT TAA GAG CAA CCT TAG CGA TTT TTA CAC TAC AAT GAC AAG TTG CTG TTT TC
T8m4	T8m4 Fwd	CGC TAA GGT TGC TCT TAA AGC TCT TAC AGC AAC TTG TCA TTG TAG TAT TCA CG
	T8m4 Rev	GCT TTA AGA GCA ACC TTA GCG ATT TTA CAA CCC ATC AGA GCT CC
T16m1	T16m1 Fwd	CCA CGT CCT CCA CAT CCA AGA TTG AAG TAA GCT TTC TTT GAA CCA AAA TTA G
	T16m1 Rev	CTT CAA TCT TGG ATG TGG AGG ACG TGG ACC ACT ACA ATG ACA AGT TGC TGT TTT C
T16m2	T16m1 Fwd	CCA CGT CCT CCA CAT CCA AGA TTG AAG TAA GCT TTC TTT GAA CCA AAA TTA G
	T16m2 Rev	CAA TCT TGG ATG TGG AGG ACG TGG TAC ACT ACA ATG ACA AGT TGC TGT TTT C
T16m3	T16m3 Fwd	CCA CGT CCT CCA CAT CCA AGA TTG TAA GCT TTC TTT GAA CC
	T16m2 Rev	CAA TCT TGG ATG TGG AGG ACG TGG TAC ACT ACA ATG ACA AGT TGC TGT TTT C
T16m4	T16m4 Fwd	CACGTCCTCCACATCCAAGATTGACAGCAACTTGTGTCATTGTAGTATTC
	T16m4 Rev	CAA TCT TGG ATG TGG AGG ACG TGG ACA ACC CAT CAG AGC TCC

**Bactericidal activity assay.** Gram-negative bacteria were incubated in 96-well microplates at 37°C overnight in the presence of nisin mutants at a final concentration of 10 times the MIC. The number of bacterial cells was standardized to a final concentration of  $5 \times 10^5$  CFU/ml. Assays with two controls in fresh LB broth with or without the bacterial inoculum were also performed in parallel under the same conditions. An aliquot was taken from each well and serially diluted in sterile phosphate-buffered saline. Afterwards, 50  $\mu$ l of each dilution was plated on LB agar plates. After overnight incubation at 37°C, the bacterial colonies were enumerated to determine the amount of viable cells.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.00052-18>.

**SUPPLEMENTAL FILE 1**, PDF file, 0.8 MB.

## ACKNOWLEDGMENTS

Qian Li is supported by the Chinese Scholarship Council (NO 201306770012). Manuel Montalbán-López was supported by grant EU FW7 from Synpeptide.

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